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STATISTICAL APPROACH TO BOAR SEMEN EVALUATION USING INTRACELLULAR INTENSITY DISTRIBUTION OF HEAD IMAGES

L. SÁNCHEZ[1†], N. PETKOV[2], E. ALEGRE[1]

[1†] Department of Electrical and Electronics Engineering, University of León, Campus de Vegazana, 24071 León, Spain
Fax: +34 98729 17 90; Email : lidia@unileon.es
[2] Institute of Mathematics and Computing Science, University of Groningen, P.O. Box 800, 9700 AV Groningen, The Netherlands

Abstract – We propose a method for the classification of boar sperm heads based on their intracellular intensity distributions observed in microscopic images. The image pre-processing comprises segmentation of cell heads and normalization for brightness, contrast and size. Next, we define a model distribution of head intracellular intensity of an alive cell using a set of head images assumed to be alive by veterinary experts. We now consider two other sets of cell head images, one formed by heads assumed to be alive by experts and another formed by cells which present some abnormalities in their cytoplasm densities and are considered as dead by the experts. We define a measure of deviation from the model intensity distribution and for each head image of the two test sets we compute the deviation from the model. While the distributions of deviation values for alive and dead cells overlap, it is possible to choose an optimal value of a decision criterion for single cell classification in such a way that the error made in the estimation of the fraction of alive cells in a sample is minimal. In the range [0.7,1.0] of interesting values of the fraction of alive cells, the standard deviation of the fraction estimation error for samples of 100 head images is smaller than 0.04. Thus, in 95% of the cases the value of the fraction of alive cells in a sample estimated by a veterinary expert will be within 8% of the estimation made according to the proposed method. This result satisfies the requirements of veterinary practice.

Key words : image analysis, classification, sperm heads, boar semen, intracellular intensity distribution, concentration of alive cells.

INTRODUCTION

Boar artificial insemination presents advantages over the natural one and it is widely used in current practice. To avoid infertility problems and to identify boars with high reproductive features, sperm quality analysis is used. Visual assessments of semen by veterinary experts or CASA (Computer Aided Sperm Analysis) systems are the classical ways to determine the potential fertility of boars. There are four factors to consider in the evaluation of boar sperm quality: concentration, motility, morphology and acrosome integrity of spermatozoa (13). In this work, we introduce a method for the automatic evaluation of acrosome integrity using digital image analysis.

Due to the complexity of sperm quality estimation, computerized techniques are essential tools. The majority of these computer methods have been developed to analyse human semen morphology and have afterwards been adapted for other species. The development of new methodologies is an ongoing research activity (4,5). These researches have enriched the available knowledge on sperm cells (15) and furthermore, digital image analysis had allowed to classify subpopulations (9) or to describe shape abnormalities (5). Most of these approaches use CASA systems (10,14) or propose new description and classification methods (1,3,6,7). The majority of them focus on spermatozoa morphology such as abnormalities relating to shape and size of sperm heads and it is very difficult to find works about vitality or acrosome integrity.

In veterinary practice, stains are used to determine if a sperm cell is alive or dead. A hypothesis of practical interest is that there is a relation between particular patterns of intracellular density distribution observed in microscopic images, sperm vitality and semen fertility. Basing on their experiences, veterinary experts assume that there is a certain correlation between the intracellular density distribution and the alive/dead status of a cell. This hypothesis has been studied with methods of digital image processing and machine learning. (2,11,12). These studies aimed at identifying a pattern that is characteristic of alive cells and localizing regions that are diagnostic for the alive/dead status of a cell. In the current work we present new results that build on this hypothesis.

The main motivation behind this and previous studies in the same direction is to explore the potential of digital image processing and machine learning for sperm fertility estimation. Beside the fundamental biological insights into the correlation between intracellular density distribution and alive/dead status of a cell that such studies can provide, they can also be of importance for veterinary practice, having a potential to substitute expensive staining techniques. The latter techniques have

certain disadvantages, such as sensitivity to temperature variations and manual manipulation of the samples, errors in pH adjustment, and relatively long stain preparation time. Some simple stain techniques used to identify alive and dead cell like eosin-nigrosin are time consuming since the incubation period is at least 5 minutes, the stain has to dry and further time is needed for evaluation. In contrast, digital image processing techniques allow to analyse 1000 or even more sperm cells in less than a minute. Another disadvantage of traditional stain procedures is their toxic effects on sperm cells that increases the number of dead spermatozoa.

In this work we adopt the following approach. First, veterinary experts, who assume that a certain intracellular density distribution is characteristic of alive cells, visually inspect microscopic boar sperm images and mark cells as alive or dead. Using a subset of images of cell heads that were marked as alive by veterinary experts we derive an intracellular density distribution model for alive cells and we define a measure of deviation of a given intracellular distribution from this model. Using this measure we assign a deviation value to each image of a cell head that has been marked as alive or dead by an expert and we use the obtained values to make a statistical inference about the error made in evaluating the fraction of alive cells according to this method.

In Methods, we present the derivation of an intracellular density distribution model, the definition of a measure of deviation from this model and the assignment of a deviation value to each cell head image from a large experimental data set. The use of the set of obtained values for evaluation of the fraction of alive cells in a sample is explained in the section Results. Finally, we summarize the results and draw conclusions.

METHODS

Semen samples collected from boars are centrifuged at 800 xg for 10 minutes. Next, the supernatant is removed and the obtained sperm pellet is diluted with MRA until a final sperm concentration of 200 millions sperm cells per ml is achieved. Finally, the sperm cells are fixed in glutaraldehide 2%. The semen sample images were acquired by means of a digital camera Nikon Coolpix 5000 connected to a phase-contrast microscope. The magnification used was 40x and the resolution of the sample images was 1600 x 1200 pixels, Fig. 1. To develop the proposed method we used Matlab and its Image Processing Toolbox.

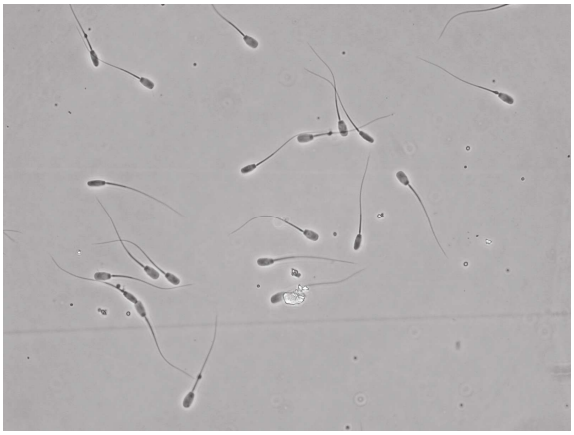

Fig 1. Example of a boar semen sample image acquired using a phase-contrast microscope.

The number of spermatozoa in an image as well as their orientation and tilt vary. A sample image can also present agglutinations of heads and debris because of the manipulation process. To isolate the sperm heads in an image, we first apply morphological closing which results in smooth head contours. Then we apply thresholding deploying Otsu's method to isolate the image regions that potentially contain heads (8). Finally, we remove those regions that are occluded by the boundaries of the image. We also do not consider isolated regions that are smaller than 45% of the average head area. This value was determined experimentally. For each sample image, the above preprocessing and segmentation steps produce an image with the isolated heads as grey level distributions on a black background, Fig. 2.

Fig 2. Image obtained from the image shown in Fig.1 by pre-processing and segmentation. Sperm heads appear as grey level distributions on a black background.

In the images obtained in this way, sperm heads appear as oval shapes with different orientations. Boar sperm heads, unlike other species, have a characteristic nearly elliptical shape. We consider the

pixels on the boundary of a head and, using principal component analysis, we compute the main axes of the ellipse that fits into the head. We use the obtained principal components of a head to rotate it and align the major and minor axes of the fitting ellipse with the x and y axes, respectively, Fig. 3.

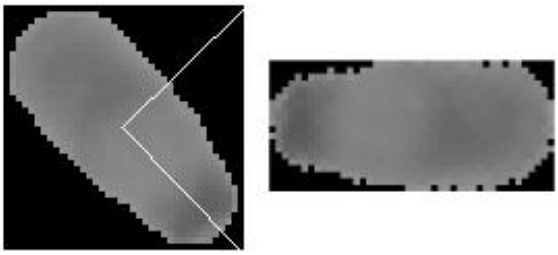

Fig 3. (left) Example of a head as it appears in the image presented in Fig.2 with the principal component axes of the best fitting ellipse and (right) a rotated head image.

The experimental measurements of sperm heads show that a normal head is from 4 to 5 μm wide and from 7 to 10 μm long. Thus the aspect ratio or the ellipse eccentricity varies from 0.4 to 0.7. As a next step we re-scale all head images to the same aspect ratio and size of 19 x 35 pixels; this re-scaling is done using nearest-neighbor interpolation. We consider a two dimensional function *f(x,y)* which is defined by the grey levels of those pixels that belong to the mentioned ellipse with minor and major axes of 19 and 35, respectively, Fig. 4.

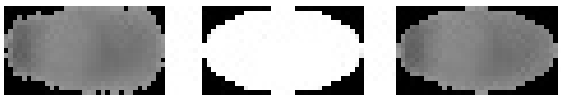

Fig 4. The rotated image is re-scaled to 19x35 pixels (left). We apply an elliptic mask of 19x35 pixels (center) in order to define the 2D grey level function *f(x,y)* (right).

Spermatozoa present different intracellular density distributions. Veterinary experts mark sperm heads as alive or dead based on their experience. Although there are considerable variations in the intracellular distributions of cells marked as alive, it is possible to distinguish three head areas according to their intensity. There are a darker area, called the post nucleus cap where the mid-piece and the tail develop from, an intermediate light area and, finally, another dark area corresponding to the acrosome which covers the anterior portion of the nucleus region.

The acrosomal status gives information about the sperm fertility since those heads that have lost their acrosomes before approaching the oocyte are unable to fertilize. To be able to fertilize, spermatozoa have to go through a capacitation process which involves several changes in the organization of the plasma membrane and the cell interior. The acrosome reaction results in a loss of the contents of the acrosome. Hence, the capacitation destabilizes the membrane to be ready for the acrosome reaction, which allows the enzymes go out of the head. The acrosome reaction entails that the plasma membrane and the acrosomal contents are lost. For this reason, the intracellular intensity distributions are not the same across different head images. Furthermore, the head images present diverse contrasts of the three mentioned regions and different head brightness averages. To deal with these latter variations, we carry out a linear transform on the grey level function *f(x,y)* of the intracellular intensity distribution in order to keep the same mean and standard deviation across all sperm head images.

Considering the 2D grey level function *f(x,y)* defined on a region *S* enclosed by the above mentioned ellipse with main axes 19 and 35 pixels, we define a linear transform of *f(x,y)* into a function *g(x,y)* defined on *S* by:

$$g(x,y) = a \cdot f(x,y) + b$$

The coefficients *a* and *b* are defined as follows:

$$a = \frac{std(g)}{std(f)}, \qquad b = \overline{g} - a\overline{f}$$

In the above formula, the values $\overline{f}$ and std(*f*) of the mean and the standard deviation of *f(x,y)* are computed directly from the function *f*.

The values of the mean $\overline{g}$ and the standard deviation std(*g*) of *g(x,y)* are fixed to 100 and 8 respectively. These target values were experimentally determined since the sperm head images assumed as potentially alive by veterinary experts take around those values for their means and standard deviations.

We now consider a set of sperm heads that have been hypothesized as potentially alive by experts based on their intracellular intensity distributions. This set contains $n = 34$ head images and it is named the *model training set M*. For each of these images we obtain an intensity distribution function $g_i(x,y)$, $i = 1 \ldots n$, as described above. Next, we define a model grey level intensity distribution function *m(x,y)* as a pixel-wise average of these functions, Fig. 5.

$$m(x,y) = \frac{1}{n}\sum_{i=1}^{n} g_i(x,y)$$

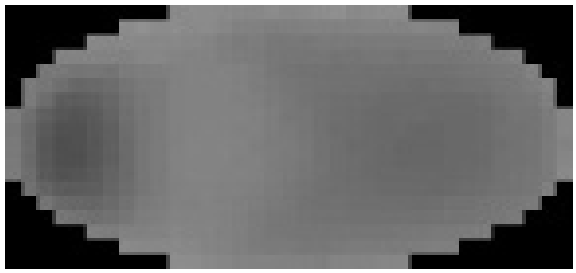

Fig 5. Model of the intracellular intensity distribution assumed as characteristic of alive sperm cells. It is computed as the average of a set of head images of sperm cells classified by experts as alive.

For each pixel which lies within the mentioned ellipse, we assess the variability of the grey levels across the model training set by computing the standard deviation *s(x,y)* for that pixel:

$$s(x,y)=\sqrt{\sum_{i=1}^{n}\frac{(g_i(x,y)-m(x,y))^2}{n}}\,.$$

Then we define and compute a deviation value *d* of the 2D grey level intensity distribution function *g(x,y)* of a given head image from the model distribution function *m(x,y)* using the infinity norm:

$$d=\max\left(\frac{|g(x,y)-m(x,y)|}{s(x,y)}\right)$$

Next we use two sets of sperm head images that have been obtained from boar semen images by applying the previously mentioned pre-processing and normalization steps. One of these sets, that we call the *alive cell set (A)*, consists of 718 heads that present intracellular intensity distributions assumed by experts as characteristic of alive sperm cells, Fig. 6. The other set, that we call the *dead cell set (D),* comprises 650 head images with intracellular intensity distributions that are assumed by experts to be characteristic of dead sperm cells, Fig. 7. For each head image from these sets we compute a function *g(x,y)* and the deviation *d* of this function from the model function *m(x,y)*.

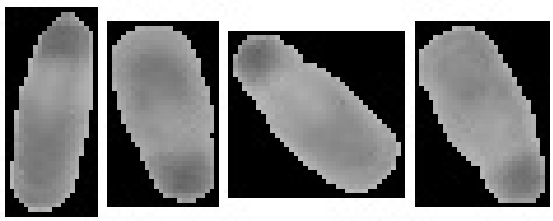

Fig 6. Examples of head images of sperm cells that were assumed to be alive by experts according to their intracellular distributions.

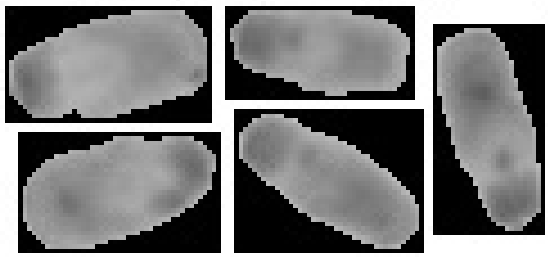

Fig 7. Examples of head images of sperm cells that were classified as dead by veterinary experts based on their intracellular distributions.

The two sets of values of the deviation *d* obtained for the two sets *A* and *D* form the basis of the further analysis.

RESULTS

Fig. 8 shows the distributions of the frequencies of occurrence of the values of the deviations obtained for the sets *A* and *D*.

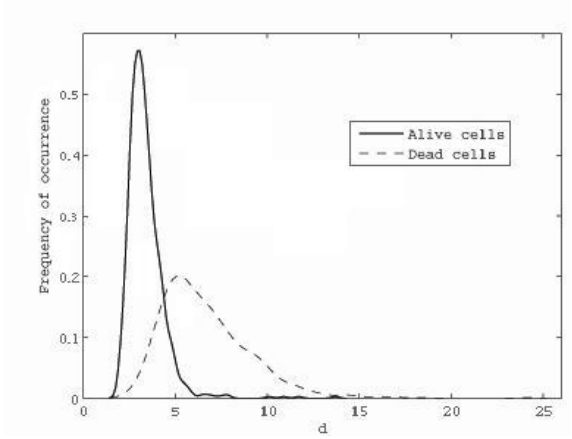

Fig 8. Frequencies of occurrence (probability densities) of alive and dead cells as functions of the deviation *d*. from the model distribution of an alive cell.

The two distributions overlap and this means that error-free classification of single cells as alive or dead is not possible for values of *d* that lie in the overlap region. Fig. 9 shows the misclassification errors obtained from the distributions shown in Fig. 8 as functions of the value d_c of a classification criterion which is used to classify cells as alive if $d \leq d_c$ and dead if $d > d_c$. For small values of d_c the false rejection error $e_r(d_c)$ (of alive cells) is high because many alive cells whose *d*-values are larger than d_c are misclassified as dead. For large values of d_c the false acceptance error $e_a(d_c)$ (of dead cells) is large because there are many dead cells whose *d*-values are smaller than d_c so that these cells are misclassified as alive.

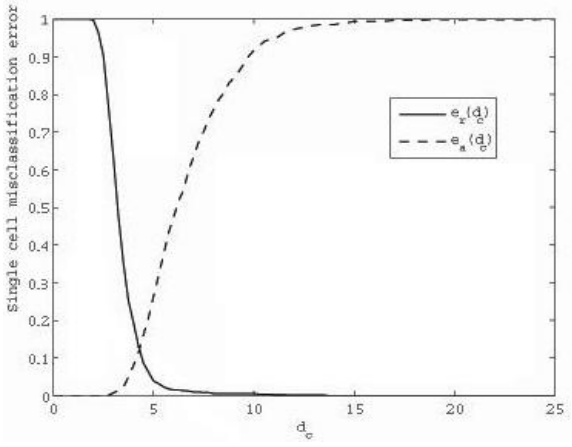

Fig 9. Misclassification errors $e_r(d_c)$ and $e_a(d_c)$ as a functions of a classification criterion d_c which is used to classify cells as alive if $d \leq d_c$ and dead if $d > d_c$. The false rejection error $e_r(d_c)$ is defined as the fraction of alive cells for which holds $d > d_c$ and which will be misclassified as dead. The false acceptance error $e_a(d_c)$ is defined as the fraction of dead cells for which holds $d \leq d_c$ and which, therefore, will be misclassified as alive.

In practice, one is interested not in the classification of single cells but rather in an estimation of the fraction p of alive cells in a given sample. When this fraction is estimated by means of single cell classification, the fractions of misclassified alive and dead cells in a very large sample will be $p \cdot e_r(d_c)$ and $(1-p) \cdot e_a(d_c)$, respectively. Note that while some alive cells are misclassified as dead, some dead cells will be misclassified as alive, and the latter number can partially compensate the former. The error in the estimation of p will thus be:

$$e(d_c,p) = | p \cdot e_r(d_c) - (1-p) \cdot e_a(d_c)|$$

and Fig. 10 shows $e(d_c,p)$ as a function of the decision criterion d_c for three different values of p. As can be seen from this figure, for each value of p the error $e(d_c,p)$ as a function of the decision criterion d_c has a minimum for a given value of that criterion and we denote this value with $d_c(p)$. This is the value of d_c for which the number of misclassified alive cells is equal to the number of misclassified dead cells so that the error in the fraction estimation is zero. Fig. 11 shows $d_c(p)$.

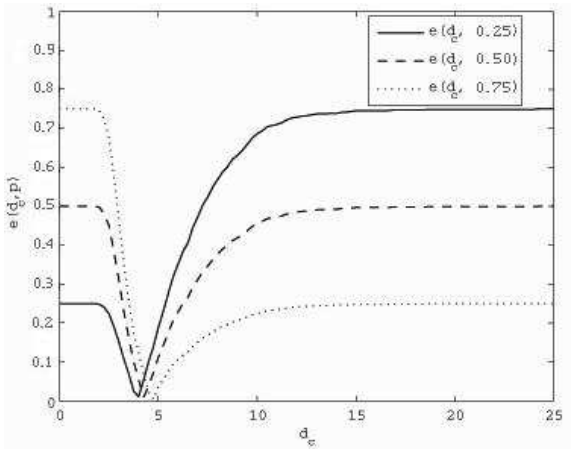

Fig 10. The error $e(d_c, p)$ in the estimation of the fraction p of alive cells in a (very large) sample as a function of the decision criterion d_c for three different values of p.

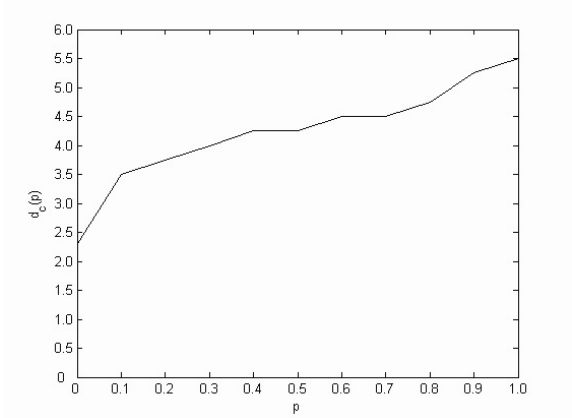

Fig 11. Value $d_c(p)$ of the decision criterion for which the error in estimating the fraction p of alive cells in a very large sample is minimal (0).

Since the fraction p of alive cells in a given sample is not known in advance, the question of how to determine the value of the decision criterion d_c that has to be applied to the head images of the cells in the sample deserves special attention. We suggest the following iterative procedure. In a first step the single cell classification is done using $d_c(0.5)$, i.e. assuming an equal number of alive and dead cells in the sample ($p = 0.5$). The result of this single cell classification delivers a first estimation p_1 of p. Now the single cell classification is repeated with a value of the decision criterion $d_c(p_1)$ and this new classification results in a new estimation p_2 of p. In practice, we found that the consecutive estimations of p converge to a stable value after only a few iterations (less than 5).

The next interesting question is how large the error is when p is estimated as proposed above. To determine this error, we take a sample of 100 head images by randomly selecting $p100$ head images from set A and $(1-p)100$ images from set D. For such a sample we estimate the fraction of alive cells according to the iterative procedure given above and we denote the resulting value by p'. The fraction estimation error $p-p'$ will be different from sample to sample. Therefore, we quantify the fraction estimation error for finite samples (of 100 head images) by the standard deviation of $p-p'$ for 100 samples. In the range $p > 0.7$ that is interesting for veterinary practice, the standard deviation of $p-p'$ is smaller than 0.04 which means that in 95% of the cases the real value of the fraction p of alive cells in a sample will be within 8% of the estimation p' made according to the method proposed above, Fig. 12. This is within the requirements of the veterinary practice.

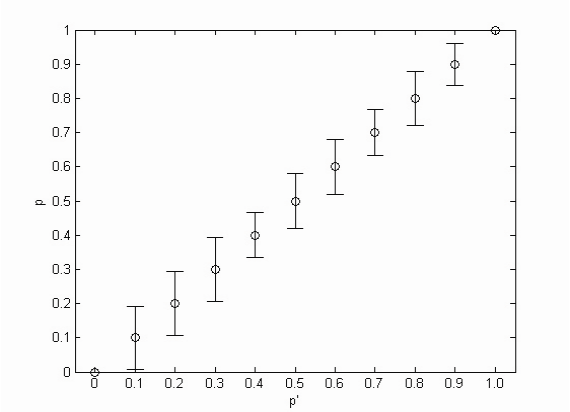

Fig 12. 95% confidence interval of the fraction of alive cells p as a function of the estimation p'.

DISCUSSION

We propose a method to classify sperm head images by means of the intracellular intensity distribution that they present. We define a model intracellular

intensity distribution that is derived from the images of heads assumed to be alive by veterinary experts. We also define a measure of deviation from this model. Using the model and the deviation measure we study the distributions of deviation values obtained for two test sets *A* and *D* of head images that are hypothesized as alive and dead by experts, respectively. Based on these distributions we make an estimation of the fraction of alive cells in a given sample and the error of this estimation. In 95% of the cases the real value of the fraction *p* of alive cells in a sample will be within 8% of the estimation *p'* made according to the proposed method. This result satisfies the requirements of veterinary practice. In future works will compare the results obtained with the proposed method with the results of stains.

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